DIFFERENTIATION OF TISSUE CULTURED HUMAN GLIOMA CELLS

by

Martin Kanje

INSTITUTE OF ZOOPHYSIOLOGY
UNIVERSITY OF LUND
LUND - SWEDEN

1977

DIFFERENTIATION OF TISSUE CULTURED HUMAN GLIOMA CELLS

by

MARTIN KANJE

UNIV. LUNDA SWE.

DOCTORAL DISSERTATION
to be publically discussed at the
Institute of Zoophysiology
University of Lund
on June 2 1977 at 1 pm

UNIV.
LEIDEN
BIBL.

CONTENTS

INTRODUCTION 4

MATERIALS AND METHODS 4

RESULTS AND DISCUSSION 6

Morphology 6

Cells in serum medium 6

Cells in serum-free medium 7

Effects of c-AMP related agents 7

RNA- and proteinsynthesis 8

Structural proteins 9

Phosphatases 10

Adhesion and aggregation properties 10

Electrophysiology 11

Calcium fluxes 11

CONCLUSIONS 12

ACKNOWLEDGEMENTS 13

FIGURES 14

REFERENCES 18

This thesis is based on the following papers, and on some unpublished results.

I Edström, A., Kanje, M. and Walum, E: Effects of dibutyryl cyclic AMP and prostaglandin E_1 on cultured human glioma cells. Exp. cell res. 85 (1974) 217-233.

II Edström, A., Kanje, M., Löfgren, P. and Walum, E.: Drug-induced alterations in morphology and level of c-AMP in cultured human glioma cells. Exp. cell res. 95 (1975) 359-364.

III Kanje, M., Walum, E. and Edström, A: Effects of dibutyryl cyclic AMP and cytochalasin B on cultured human glioma cells. Cell and tissue res. (submitted).

IV Kanje, M: Phosphatase activity in cultured glioma and neuroblastoma cells. Exp. cell res. (1977) in press.

V Arlock, P. and Kanje, M: Effects of dibutyryl cyclic AMP on the transmembrane potentials of cultured human glioma cells. Exp. cell res. (1977) in press.

VI Lazarewicz, J.W., Kanje, M., Sellström, A. and Hamberger, A.: Calcium fluxes in cultured and bulk isolated neuronal and glial cells. J. neurochem. (1977) in press.

The papers will be referred to by their Roman numbers.

INTRODUCTION

In 1971 the c-AMP analogue, dibutyryl cyclic AMP, was shown to restore several characteristics of normal cells to transformed sarcoma- and Chinese hamster ovary cells (Johnson et al., 1971; Hsie & Puck, 1971; Hsie et al., 1971). The process was termed "reversed transformation" implicating its importance. Further studies have shown that other neoplastic cell-lines, including cells of neuronal origin,differentiate after treatment with c-AMP analogues or agents which increase their c-AMP content (Prasad & Hsie, 1971; Prasad, 1972 a, b; Prasad & Sheppard, 1972; Macintyre et al., 1972; Prasad & Kumar, 1973).

The majority of tumors, if not all, which arise in the central nervous system are of glial origin (Pontén, 1973). This study is concerned with the effects of c-AMP related agents on cultured human glioma cells and their usefulness as a model system for glia cell function. Cultured neuroblastoma cells are well characterized (Schubert, 1971; Sato, 1973) and have been used as a reference system in some studies.

MATERIALS AND METHODS

Materials and methods are here summarized. Readers are referred to original articles for details.

Cells

An established line of human glioma cells, 138 MG, derived from a grade III astrocytoma-glioblastoma, isolated by Pontén and Macintyre 1968, was used. The glial origin of these cells is supported by their content of S-100 and glial fibrillary acidic protein (Edström et al., 1973; Walum, 1975).

The neuroblastoma cells employed in this study was a clone, 41A3, of the C 1300 cell-line established from a mice tumor by Augusti-Tocco and Sato, 1969.

Culture conditions

Stock cultures were grown in glass or plastic flascs. In most experiments Falcon 60 mm vented plastic Petri dishes were used as cell substrate. Ham's F 10 medium (Ham, 1963), supplemented with penicillin, streptomycin, amphotericin B and 10% new-born calf serum, was used for the glioma cells. The medium employed for neuroblastoma cells contained an additional 5% fetal calf serum.

The cultures were kept at 37°C in a humidified atmosphere of 5% CO_2 in air.

Subcultures were made by the use of 0.15 - 0.25% trypsin in an isotonic sodium phosphate buffer.

Cell number was determined in a 401 Celloscope by counting cells removed by trypsin.

Cell viability was measured by incubation of cells with 0.9% trypan blue in Hanks' solution. The cells which stained blue were regarded as non viable.

Morphological studies

Most morphological experiments were performed on cultures around confluency (approx 50 0000 cells/cm^2 culture dish). The cells were washed prior to incubation with media ± drugs. The cellular morphology was studied at different time intervals with a light microscope. In some experiments cells exposed to drugs were fixed, stained with Giemsa solution and photographed. Conventional transmission and scanning electron microscopical techniques were employed for the study of the ultrastructure of the cells.

Determination of c-AMP

Cyclic AMP was measured on confluent cells incubated for various periods of time with or without test agents in serum-free medium. Following washing with ice-cold Hanks' solution c-AMP was extracted with trichloroacetic acid. The c-AMP content of the extract was determined by the Mannheim-Boehringer radiometric test for c-AMP (Gilman, 1970; Brown et al., 1971).

Determination of protein

Protein was determined on cellular material dissolved in 1 M NaOH according to Lowry et al., 1951.

Phosphatase activity

Glioma and neuroblastoma cells were removed from their substrate and homogenized in deionized detergent containing water. The phosphatase activity of the homogenates was determined using paranitrophenol as substrate as described by Hess & Pope (Colowick & Kaplan, 1957).

Cell movement

The movement of glioma cells were assayed by their ability ot invade a cell-free scratch made in the confluent layer of cells with a glass needle.

The scratches were photographed and observed at the beginning of the experiment and after 12 h incubation with serum containing medium ± drugs.

Adhesion and aggregation tests

Glioma cells around confluency were exposed to fresh medium ± drugs for 2 h at 37°C. The culture medium was then replaced by Ca^{2+} and Mg^{2+}-free Hanks' solution with 5 mM EDTA. The cultures were placed on a gyratory shaker (100 rpm). The number of detached cells was determined after 15 min. Adhesion was expressed as removed cells in per cent of the total number of cells.

Cells for aggregation tests were exposed to medium ± drugs for 24 h. The cells were then removed by incubation with Ca^{2+}- and Mg^{2+}-free Hanks' solution with EDTA. The cells were collected by centrifugation and washed twice in a complete Hanks' solution. Finally the cells were suspended in an appropriate volume of Hanks' solution with 1 mg concanavalin/ml for 15 min at room temperature. Aliquotes of the suspensions were observed and photographed through a dark field microscope.

Electrophysiology

Transmembrane potentials of cells treated with various media were recorded at 20°C in Hanks' solution. Potentials were obtained through 3 M KCl filled glass electrodes which were micromanipulated into the desired cells under an inverted microscope utilizing conventional electrophysiological techniques.

Uptake and efflux studies

The uptake of hexoses was assayed by the use of ^{3}H -2-deoxy-D-glucose as described by Walum, 1975. Calcium fluxes were measured in the cells utilizing ^{45}Ca. Radioactivity was determined by standard liquid scintillation techniques.

RESULTS AND DISCUSSION

MORPHOLOGY

Cells in serum-containing medium

The 138 MG cell morphology is different at different cell densities in serum containing medium. At low densities, below 50 000 cells/cm^2, most cells lack processes and have a flattened irregular morphology (I). The

cells have a smooth surface with few villis, 1 - 3 µm in length (III). The majority of cells contain one nucleus with 1 - 3 nucleoli. In suspension the cells have an average diameter of 20 µm. Less than one per cent of the cells are multinuclear. The latter cells have a diameter of 40 µm or more in suspension.

At high densities, 50 000 - 100 000 cells/cm^2, the cells become more or less spindle-shaped. The surface of these cells is wrinkled and has an increased number of villis.

At still higher densities star-shaped cells can be observed in scratches made in the bottom of the dishes (Edström et al., 1976). The variation in morphology observed in sparse and dense cultures is probably to some extent dependent on the availability of substrate to spread on.

Cells in serum-free medium

Low density 138 MG cells deprived of serum obtain a bipolar morphology (I). These cells show a wrinkled surface and an increased number of villis (III). In the absence of serum the cells are growth inhibited (Edström et al., 1975) and an increase of S-100, a protein characteristic of the nervous system, occurs (Edström et al., 1973). Glial tumors contain lower levels of S-100 protein than normal glia cells (Haglid et al., 1973). It is possible that serum-deprived glioma cells exhibit some features of differentiated glial cells. In neuroblastoma cells serum deprivation induces neurite extension (Schubert et al., 1971), another expression of some normalization process.

Effects of c-AMP related agents

Dibutyryl cyclic AMP (dbc-AMP), but not butyric acid or sodium butyrate, in serum medium within some hours induced an astrocyte-like morphology to the cells (I; V). Other c-AMP analogues, N^6- Δ^2-isopentyl -adenine(DMA) and zeatin caused similar changes (II). The cell body rounded up och processes of various lengths with a diameter of 1 - 3 µm were formed by retraction of the cell margin. On prolonged incubation (1 - 3 days) the processes grew and elongated. The number of processes per cell varied strongly and some cells showed 20 or more processes. The cell surface was folded and had motile villis which also could be seen on the processes. This morphology can not, at the light microscopical level, be distinguished from that of normal astrocytes in histological sections (Glees, 1955). A stimulatory action of the c-AMP analogues on phosphokinases (Johnson et al., 1974) is a probable explanation to their present effects.

Phosphodiesterase inhibitors like papaverine, RO 20 1724 and RO 20 7222 induced similar morphological changes (II). These were accompanied by an increased level of c-AMP in the presence of the two former drugs (II). Another well-known phosphodiesterase inhibitor, theophylline, did not affect the morphology (I). However, neural tissue differs from other tissues in response to phosphodiesterase inhibitors. Theophylline, for instance, does not influence the c-AMP content of brain-slices or astrocytoma cells (Kakiuchi et al., 1969; Clark et al., 1971).

Of several neurotransmittors tested only adrenergic ones were found to induce an astrocyte-like morphology and an increased content of c-AMP. The effect could be blocked by the adrenergic β-blocking agent sotalol, which suggests the presence of a β-receptor associated adenylate cyclase. A nutritional relationship between nerve and glial cells, involving release of catecholamines from neurons, stimulation of adenylate cyclase in glial cells with concomitant efflux of glucose by c-AMP stimulated glycogenolysis, has been suggested (Newburgh et al., 1972). It is in this context interesting that the transport of glucose analoques increases in dbc-AMP treated 138 MG cells (Edström et al., 1975).

Prostaglandin E_1 (PGE_1), another well-known adenylate cyclase stimulator, was also found to induce an astrocyte-like morphology and increase the c-AMP content of the glioma cells (I, II).

The morphological alterations were potentiated in serum-free medium (I). An increase in c-AMP after serum deprivation, which has been demonstrated in neuroblastoma cells (Ryan & Heidrick, 1974) and also in 138 MG cells (Löfgren, personal communication) may account for the potentiating effects of serum-free medium.

The present data suggest that c-AMP has an important role for morphological "normalization" of glioma cells. It is interesting that astrocyte-like cells are formed after treatment of fetal brain cell cultures with monobutyryl cyclic AMP (Shapiro, 1971) which could imply that an increased c-AMP level is the physiological signal for differentiation of normal glia cells.

RNA- and protein synthesis

Actinomycin-D, a well-known inhibitor of RNA-synthesis, did not affect the morphological alterations induced by dbc-AMP. Cycloheximide, which effectively inhibits protein synthesis in the present system (I), did not inhibit the formation of stellate cells but suppressed growth and elongation of the processes. Accordingly the morphological changes are largely controlled

at a posttranslational level,probably by reorganization of available components. Similar studies on neuroblastoma cells show that initial formation of neurites requires protein- but not RNA-synthesis (Prasad & Hsie, 1971). The difference between glioma and neuroblastoma cells with regard to dependence on protein synthesis is probably due to their different modes of process formation (Steinbach & Schubert, 1975). The latter cells actively grow out extensions from the cell body whereas glioma cells form processes by retraction of the cell margin.

Structural proteins

Microtubules

Microtubules (MT) are only occasionally seen in electron microscopical pictures of undifferentiated glioma cells but are prominent in processes of dbc-AMP treated cells (Fig. 1 & 2). Vinblastine and colchicine, known to interfere with MT, inhibited the morphological alterations caused by dbc-AMP and destructed already formed processes (I, III). Assembly of MT from presynthesized subunits therefore seems to be a prerequisite condition for the morphological changes of glioma cells. This is also the case for neuroblastoma cells and Chinese hamster ovary cells (Hsie & Puck, 1971; Prasad & Hsie, 1971; Kirkland & Burton, 1972; Puck et al., 1972). No effects of c-AMP on polymerization of MT in cell-free solution have been observed, which does not exclude the importance of c-AMP for the assembling of MT in the more complex cell system. For instance, an increased number of MT can be observed with immuno-histochemical techniques in dbc-AMP treated cells (Frankel, 1976).

Microfilaments

Cytochalasin B (CB) sensitive 60 Å filaments, microfilaments (MF), have been demonstrated in cultured glia cells (Spooner et al., 1971). They are thought to be involved in contractile events like cell-movement and cell--division (Carter, 1967) but may also play an important role for maintenance and changes in cell morphology (Willingham & Pastan, 1975). These events are affected by CB, which is furthermore known to inhibit carrier mediated glucose transport in the 138 MG cells (Walum, 1975) like in other cells (Zigmond & Hirsch, 1972).

CB induced a stellate morphology to the glioma cells, strongly reminding, but easily distinguished from that of dbc-AMP treated cells (III). Also with respect to cell-movement, cell-division and uptake of hexoses, CB and dbc-AMP exerted similar effects (III). The effects of CB implicate a role for MF in the morphological changes of the glioma cells. It

is tempting to speculate that c-AMP affects the same cellular structure.

Glial fibrils

Glial fibrils with a diameter around 60-90 Å and presumably composed of glial fibrillary acidic protein (GFA) (Dale et al., 1975) are numerous in the 138 MG cell cytoplasm (Fig. 1 & 2). Their role is unknown but it lies near at hand to suggest a skeleton function. A stellate network,which stains with anti-GFA,can be seen in cultured astrocytes (Dale et al., 1975). A network of similar appearance can be observed with phase contrast microscopy of dbc-AMP treated 138 MG cells (unpublished observation).

Phosphatases

The activation of proteinkinases and subsequent phosphorylation of proteins probably accounts for most c-AMP mediated responses (Greengard & Kuo, 1970). Phosphatases could, by dephosphorylation, affect c-AMP mediated phosphorylation. The phosphatase activity of the glioma and neuroblastoma cells and its relation to the morphological changes were therefore investigated (IV).

Glioma and neuroblastoma homogenates contained phosphatase activity with pH maximum around 5. Neuroblastoma exhibited an additional maximum at pH 8. The acid phosphatase activity of both cell-lines was inhibited by NaF, $CuCl_2$ and tartaric acid and showed in these respects properties characteristic of phosphatase activity of other neural tissues (Myrbäck et al., 1961).

Cyclic GMP stimulated the acid phosphatase activity of both glioma and neuroblastoma cells. The inhibitors and c-GMP alone or in combination with dbc-AMP failed to affect the morphology of the glioma and neuroblastoma cells. It therefore appears as if acid phosphatase activity and c-AMP induced changes in morphology are interdependent. However, attention must be paid to the possibility of different actions of enzyme inhibitors and stimulators in homogenates and on intact cells. It should be noted that neither the extracellular presence of acid nor alkaline phosphatase affected dbc-AMP induced alterations in morphology (Kanje, unpublished).

ADHESION AND AGGREGATION PROPERTIES

As compared to normal cells neoplastic ones are easier to remove from their substrate by EDTA and form larger aggregates in the presence of plant lectins e.g. concanavalin A (Johnson & Pastan, 1972 ; Inbar & Sachs, 1969). Table 1 and Fig. 3 demonstrate the adhesion and agglutination properties of the 138 MG cells. Cells exposed to dbc-AMP or agents which increase their c-AMP

content (II) were less easily detached than control cells. Cells exposed to dbc-AMP, but not to butyric acid, formed smaller aggregates in the presence of concanavalin A.

The results suggest that c-AMP induces fundamental changes of the glioma cell surface of a kind which can be interpreted as a normalization process.

ELECTROPHYSIOLOGY

Little is known about the electrophysiological properties of glial cells, which possibly play a role to regulate the extra-cellular milieu of the nerve cell during excitation. Mammalian glial cells in vivo have a mean transmembrane potential of about -90 mV (Dennis & Gerschenfeld, 1969; Krnjević & Schwartz, 1967; Ransom, 1974). The potentials were found to be lower in primary cultures of glial cells (Hild et al., 1958; Hild & Tasaki, 1962) and still lower in cultured rat glioma cells (Hamprecht et al., 1976). The difference could reflect a correlation between membrane potential and the degree of differentiation.

The transmembrane potentials of 138 MG cells were around -35 mV (V). After treatment with dbcAMP the potentials increased to -40-50 mV. The increase did not occur in cells exposed to butyric acid. The membrane potential was potassium dependent and it can be assumed that the dbc-AMP induced increase is due to an increased intracellular K^+ concentration.

The increase in transmembrane potential is most likely ascribed to changed membrane properties. Whether or not the electrical changes as such reflect a normalization process is an open question. The preparation could offer experimental possibilities to obtain a better understanding of the electrophysiological function of glial cells.

CALCIUM FLUXES

It is apparent that several aspects of cellular function can be studied in cultured neoplastic neuronal cells. Whether or not such cell systems also possess the specificities of their original cell-type, i.e. could be used as model systems for the normal function of nerve and glial cells, is not always obvious. It therefore seems important to study the properties of neuroblastoma and glioma cells in relation to those of isolated normal nerve and glia cells.

Calcium fluxes in the nervous system are closely connected with several aspects of neural function for instance transmission of impulses and

neurotransmittor release (Katz and Miledi, 1967; Kostyuk et al., 1974).

Paper VI describes calcium fluxes of bulk isolated nerve and glia cells as compared to those of neuroblastoma and glioma cells. The results showed several similarities of calcium fluxes and their ionic regulation between on the one hand neural and neuroblastoma cells and on the other hand glia and glioma cells. It has previously been shown that glioma (138 MG) and neuroblastoma (C 1300) cells are good models of glia and nerve cells with respect to transport of hexoses (Walum, 1975).

CONCLUSIONS

Cultured human glioma cells, treated with derivatives of c-AMP or agents which increase their content of c-AMP, aquire several properties with respect to morphology, proliferation, transmembrane potential, adhesion and aggregation, which characterize normal glial cells. Untreated cells retain specific properties of Ca^{2+} transport of normal glial cells.

It appears as if 138 MG cells in several ways are good representatives of normal glial cells. However, like other neoplastic cell-lines, glioma cells are probably dedifferentiated in several ways. They are kept in an artificial milieu and are isolated from normal interactions with other cells. The latter is not least of great importance for nervous functions. These aspects must be taken into consideration when results from studies on glioma cells and their relevance for normal glial function are discussed.

ACKNOWLEDGEMENT

This work has been carried out at the departments of Zoophysiology in Lund and Göteborg. I would like to express my gratitude to both departments, which by offering their facilities, have made this study possible. Special thanks are due to;

Prof. Anders Edström, who initiated this work, for his co-operation and valuable criticism during my studies.

Prof. Ragnar Fänge for stimulating discussions and providing excellent working conditions in Göteborg.

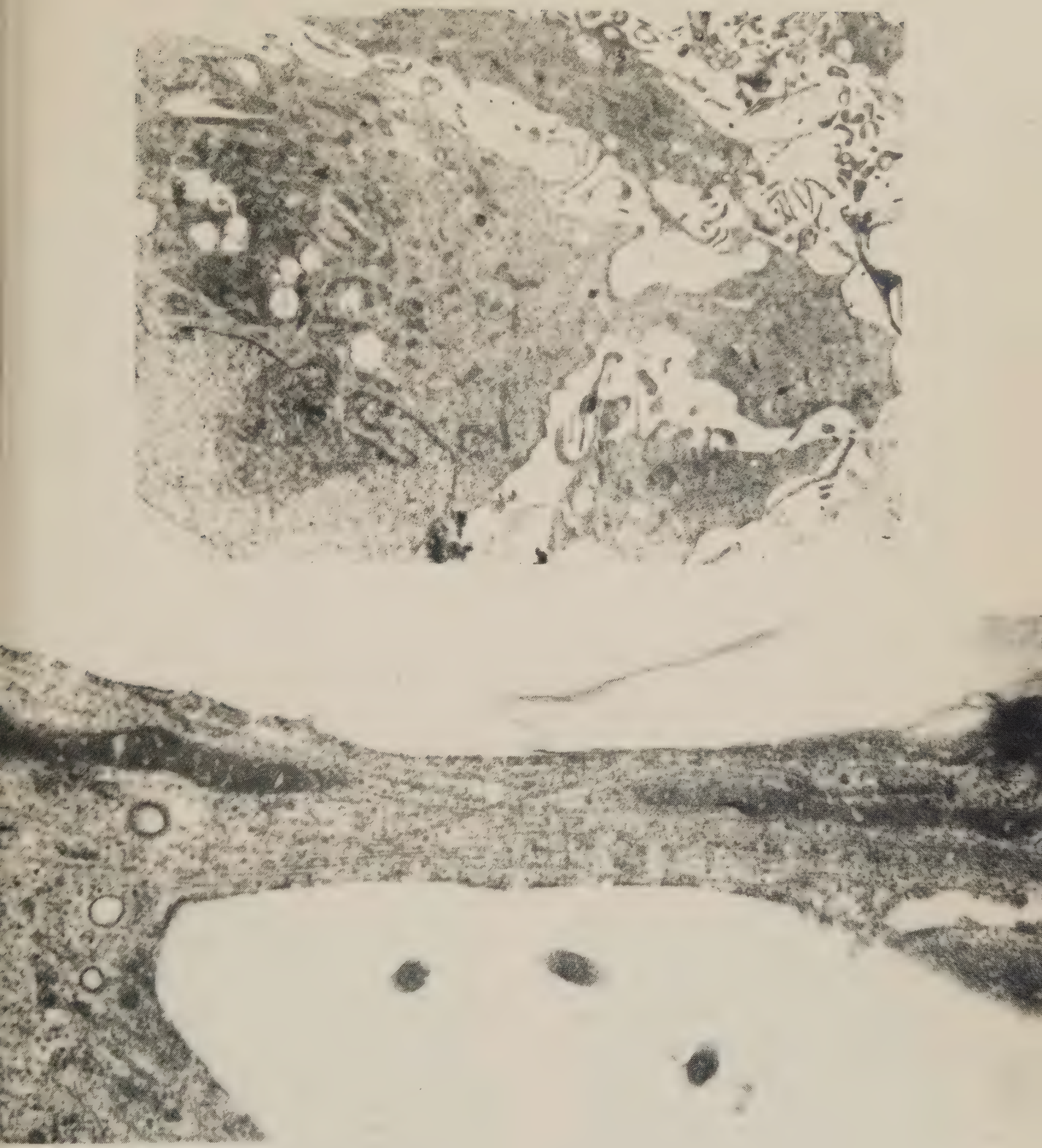
My co-authors and collaborators; Per Arlock, Lars-Johan Erkell, Anders Hamberger, Jerzy Lazarewicz, Peter Löfgren, Åke Sellström and Erik Walum.

Eva Dahlberg, Barbro Egnér, Eva Fjellstedt, Monika Lindhé, Peggy Petersen and Aina Stenborg for expert technical assistance.

Britt Claesson for cleaning utensils.

Maj-Britt Wallgren for administration of grants.

Marie Adler-Maihofer, Marianne Andersson and Agneta Gustavsson for typing manuscripts.



ELECTRON MICROSCOPICAL PICTURES OF 138 MG CELLS

Fig 1 (Top) cells kept in serum. Magnification 8000 X

Fig.2 (Bottom) process of cell exposed to dbc-AMP. Magnification 50 000 X.

The cells were fixed in formaldehyde/glutaraldehyde, postfixed in OsO_4, dehydratet in a series of ethanol solutions, embedded in Epon, sectioned and "stained" with uranyl acetate and lead prior to examination in the electron microscope.

TABLE 1 ADHESION OF 138 MG

Treatment		per cent detached cells
Serum medium (S)		[a]49.2 ± 10
S + Na-butyrate	1.0 mM	45 ± 5
S + dbc-AMP	1.0 mM	9 ± 2
Serum free medium (SF)		41 ± 8
SF + dbc-AMP	1.0 mM	9 ± 2
SF + Adrenalin	0.1 mM	8 ± 2

AGGLUTINABILITY OF 138 MG CELLS

Darkfield micrographs of suspensions of glioma cells treated for 24 h with 1 mM sodium-butyrate in serum medium (Fig. 3) and with 1 mM dbc-AMP (Fig. 4). Cell aggregates are visible as white spots.

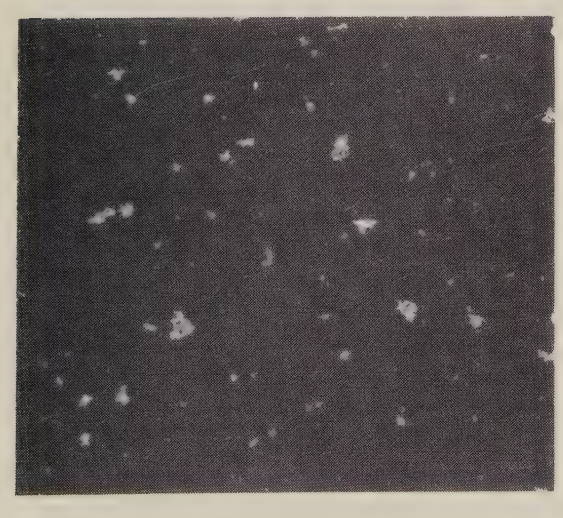


Figure 3

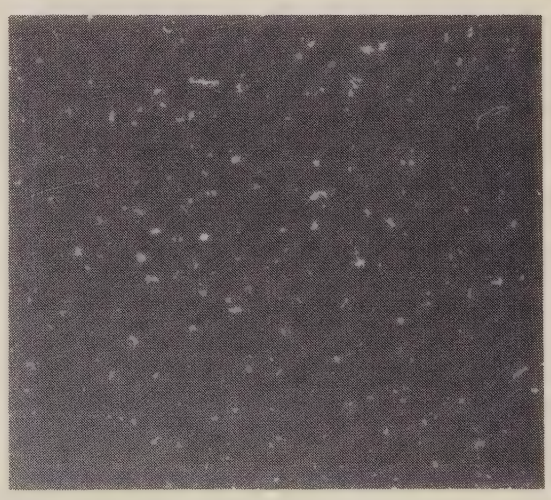


Figure 4

REFERENCES

Augusti-Tocco, G. & Sato, G.: Proc. natl. acad. sci. 64 (1969) 311.

Brown, B.L., Albano, J.D.M., Etkins, R.P., Sgherzi, M. & Tampion, W.: Biochem. J. 121 (1971) 561.

Carter, S.B.: Nature 213 (1967) 261.

Clark, B.C. & Perkins J.P.: Proc. natl. acad. sci. 68 (1971) 2757.

Colowick, S.P. & Kaplan, N.O.: Methods in enzymology IV, Academic Press, New York and London, 1957.

Dale, S.A., Choi, H.B., Lapham, L.W.: Brain res. 89 (1975) 363.

Dennis, M.J. & Gerschenfeld, H.M.: J. physiol. 203 (1969) 211.

Edström, A., Haglid, K.G., Kanje, M., Rönnbäck, L. & Walum, E.: Exp. cell res. 83 (1973) 426.

Edström, A., Kanje, M. & Walum, E.: J. neurochem. 24 (1975) 395.

Edström, A., Kanje, M. & Walum, E.: Exp. cell res. 97 (1976) 6.

Frankel, R.: Proc. natl. acad. sci. 73 (1976) 2798.

Gilman, A.G.: Proc. natl. acad. sci. 67 (1970) 305.

Gilman, A.G. & Nirenberg, M.: Nature 234 (1971) 356.

Glees, P., Neuroglia: morphology and function, Blackwell, Oxford, 1955.

Greengard, P. & Kuo, J.F.: Advances in biochemical psychopharmacology III, Raven Press, New York, 1970.

Haglid, K.G. & Stavrou, D.: J. neurochem. 20 (1973 a) 1523.

Haglid, K.G., Stavrou, D., Rönnbäck, L., Carlsson, C.A. & Weidenback, W.: J. neurol. sci. 20 (1973 b) 103.

Ham, R.G.: Exp. cell res. 29 (1963) 515.

Hamprecht, B., Kemper, W. & Amano, T.: Brain res. 101 (1976) 129.

Hild, W., Chang, J.J. & Tasaki, I.: Experentia 14 (1958) 220.

Hild, W. & Tasaki, I.: J. neurophysiol. 25 (1962) 277.

Hsie, A.W. & Puck, T.T.: Proc. natl. acad. sci. 61 (1971) 1123.

Hsie, A.W., Jones, C. & Puck, T.T.: Proc. natl. acad. sci. 68 (1971) 1648.

Inbar, M. & Sachs, L.: Proc. natl. acad. sci. 63 (1969) 1418.

Johnson, G.S., Friedman, R.M. & Pastan, I.: Proc. natl. acad. sci. 68 (1971) 425.

Johnson, G.S. & Pastan, I.: Nature new biol. 236 (1972) 247.

Johnson, G.S., Darmiento, M. & Carchman, R.A.: Exp. cell res. 85 (1974) 47.

Kakiuchi, S., Rall, T.W. & McIlwain, H.J.: J. neurochem. 16 (1969) 485.

Katz, B. & Miledi, R.: Nature 215 (1967) 651.

Kirkland, W.L. & Burton, P.R.: Nature new biol. 240 (1972) 205.

Krnjević, K. & Schwartz, S.: Exp. brain. res. 3 (1967) 306.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. & Randall, R.J.: J. biol. chem. 193 (1951) 265.

Macintyre, E.H., Wintersgill, C.J., Perkins, J.P. & Vatter, A.E.: J. cell. sci. 2 (1972) 639.

Myrbäck, K., Boyer, P.D. & Lardy, H.: The enzymes V, Academic Press New York and London, 1961.

Newburgh, R.W. & Rosenberg, R.N.: Proc. natl. acad. sci. 69 (1972) 1677.

Pontén, J., Macintyre, E.H.: Acta pathol. microbiol. scand. 74 (1968) 465.

Pontén, J.: Tumörpatologi, Almquist ° Wiksell, Stockholm, 1973.

Prasad, K.N. & Hsie, A.W.: Nature new biol. 233 (1971) 141.

Prasad, K.N.: Nature new biol. 236 (1972 a) 49.

Prasad, K.N.: Proc. soc. exp. biol. med. 140 (1972 b) 126.

Prasad, K.N. & Sheppard, J.R.: Exp. cell res. 73 (1972) 436.

Prasad, K.N. & Kumar, S.: Proc. soc. exp. biol. med. 142 (1973) 406.

Puck, T.T., Waldren, C.A. & Hsie, A.W.: Proc. natl. acad. sci 69 (1972) 1943.

Ransom, B.R.: Brain res. 69 (1974) 83.

Ryan, W.L. & Heidrick, M.L.: Advances in cyclic nucleotide res. 4 (1974) 81.

Sato, G.: Tissue culture of the nervous system, Plenum Press, New York (1973).

Schubert, B., Humpreys, S., DeVitry, F. & Jacob, F.: Dev. biol. 25 (1971) 514.

Shapiro, D.L.: Nature 241 (1971) 203.

Spooner, B.S., Yamada, K.M. & Wessels, N.K.: J. cell biol. 49 (1971) 595.

Steinbach, J.H. & Schubert, D.: Exp. cell res. 91 (1975) 449.

Walum, E.: Uptake of glucose into cultured tumor cells from the nervous system. Thesis, Göteborg, 1975.

Willingham, M.C. & Pastan, I.: J. cell biol. 67 (1975) 146.

Zigmond, S.H. & Hirsch, J.G.: Science 176 (1972) 1432.